Effects of Male Accessory Gland Proteins on Female Reproductive Physiology in the Northern House Mosquito, *Culex pipiens*

Research Thesis

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By

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Abstract

During the summer, females of the Northern House Mosquito, *Culex pipiens*, transmit West Nile virus to humans, while during the winter females stop biting humans and enter a dormant state. Male mosquitoes produce proteins in their accessory glands (MAGs) that are transferred to females during copulation. MAG proteins and other components of the ejaculate alter female behavior and physiology by increasing biting propensity and bloodmeal digestion, enhancing fecundity, and facilitating sperm survival in the female reproductive tract, all of which contribute to female reproductive success and disease transmission. Our earlier work suggests that males of *Cx. pipiens* differentially regulate several genes in their MAGs in response to seasonal cues. Specifically, *Cathepsin-B-like thiol protease* (*CapB*) and *Trypsin-1 precursor* (*Tryp1*) were upregulated in the MAGs of long day-reared males, and we hypothesize that this increases female biting propensity and fecundity. In contrast *Glutathione-S-transferase* (*GST*) was upregulated in the MAGs of short day-reared males, and we hypothesize that this may increase sperm survival in overwintering females. To test our hypotheses, we first attempted to confirm that these genes were differentially expressed in the MAGs of *Cx. pipiens* that were reared under long day, summer-like conditions and short day, winter-like conditions. Next, we used RNA interference (RNAi) to knock down these genes of interest in males, then allowed them to mate with virgin females and observed effects. We anticipated that knocking down *CapB* and *Tryp1* in males would decrease the likelihood that mated females will take a blood meal and that these females will produce fewer offspring. In contrast, we predicted that knocking down *GST* in males would result in lower sperm survival in overwintering females. Our results showed that *CapB, Tryp1* and *GST* are not differentially expressed in long day vs short day-reared males. We found that injecting males with dsRNA had variable effects on the transcript levels of

targeted genes, reducing *GST* mRNAs but increasing *CapB* mRNA. Females that mated with males injected with *CapB* dsRNA were less likely to take a blood meal. We were unable to significantly knock down *Tryp1* in males. Although females that mated with *Tryp1* dsRNA-injected males showed no significant differences in the number of eggs laid or larvae hatched, they did lay eggs that fell apart more easily than other groups. Our results suggest that MAG-derived *CapB* and *Tryp1* have effects on female reproductive behavior and physiology.

1. Introduction

Mosquitoes are effective vectors of human diseases and are considered to be the deadliest animals in history (WHO 2020). Only female mosquitoes bite humans and animals, therefore they are able to transmit vectors that cause diseases such as malaria, dengue, Zika and others that claim over 700,000 lives annually (WHO 2020). West Nile virus, primarily transmitted by mosquitoes in the genus *Culex*, is the most prevalent mosquito-borne disease in the United States. In 2018 alone, there were 2,647 cases of West Nile virus in humans and 167 deaths from the disease in the United States (CDC 2018). To date, the most effective way to limit the spread of mosquito-borne diseases is to target the mosquitoes themselves (Dahmana and Mediannikov 2020). For example, releasing transgenic yellow fever mosquitoes, *Aedes aegypti*, has proven to be effective at reducing population numbers (Carvalho et al. 2015), and models for West Nile virus suggest that reducing populations of the Northern house mosquito, *Culex pipiens*, will lead to a decrease in human cases (Bowman et al. 2005).

During mating, male mosquitoes transfer components of their seminal fluid that have physiological and behavioral effects on females (reviewed by Avila et al. 2011, Roy et al. 2016, South and Catteruccia 2016). Male accessory glands (MAGs) are organs in insects that produce

47 hormones and proteins that contribute to sperm survival, storage and motility within the female
48 reproductive tract (Boes et al. 2014). In the yellow fever mosquito, *Aedes aegypti*, a peptide
49 from the seminal fluid was found to modulate female host-seeking and inhibit subsequent mating
50 post-copulation (Lee and Klowden 1999). Male seminal components were also found to increase
51 female longevity and blood feeding propensity in *Ae. aegypti* (Villarreal et al. 2018). Male
52 accessory gland proteins that are transferred to females during copulation also affect female
53 sexual refractoriness, blood feeding behavior and blood meal digestion in *Ae. aegypti* (Downe
54 1975). Transplanting male accessory glands (MAGs) into females of *Cx. pipiens* resulted in
55 females not being inseminated when exposed to mates, indicating that MAG products also cause
56 females of *Cx. pipiens* to become refractory to re-mating (Craig 1967). Two genes, *Trypsin-1*
57 *(Tryp1)* precursor and *cathepsin-B-like thiol protease*, show increased expression in females of
58 the malaria mosquito, *Anopheles gambiae*, and *Ae. aegypti* after taking a blood meal and are
59 believed to promote blood meal digestion after mating (Dana et al. 2005). However, it is unclear
60 whether these proteins are transferred to females during copulation, and whether they influence
61 the female's ability to digest and process a blood meal. In the Asian tiger mosquito, *Ae.*
62 *albopictus*, *Glutathione S-Transferase* (*GST*) is a MAG-produced seminal fluid protein that is
63 transferred to females and is hypothesized to increase sperm survival in the females by protecting
64 them from oxidative stress (Boes et al. 2014). Further research into the gene expression and
65 transfer of seminal proteins in *Cx. pipiens* could provide valuable insight on how changes in gene
66 expression within MAGs might affect female physiology and behavior after mating.
67 Unlike tropical mosquitoes such as *An. gambiae* and *Ae. aegypti*, females of *Cx. pipiens*
68 show marked differences in behavior and physiology as seasons change, actively blood feeding
69 and laying eggs during the summer and entering an overwintering dormancy or diapause in the

winter where they do not blood feed or lay eggs (Mitchell 1983). Females enter their overwintering dormancy in response to the short days of late summer and early fall (Spielman and Wong 1973, Sanberg and Larsen 1973). Males do not enter diapause and die shortly after mating with females in the fall (Spielman 2001), leaving females to store sperm and keep it viable for the duration of winter.

Little research has been done on whether or not MAG gene expression changes in response to seasonal cues (reviewed by Meuti and Short, 2019), as well as how MAG gene expression affects female mosquitoes. Males of *Cx. pipiens* reared under long-day conditions showed high levels of expression of *Trypsin-1* precursor (*Tryp1*) and *cathepsin-B-like thiol protease* (*CapB*), while males reared under short-day conditions showed high levels of expression in the *glutathione S transferase* (*GST*) gene (Meuti lab, unpublished data). It is unclear whether or not the proteins these genes code for are transferred to females, and if they are, what effects these seminal fluid proteins may have on females after copulation. Further research on the role of these genes could provide insight as to how seasonal changes in gene expression in male mosquitoes influences fecundity and sperm survival in females. Here, we first attempted to confirm differential gene expression between independent samples of long day and short day-reared males using qRT-PCR (quantitative Real Time PCR). Then, we use RNAi to knock down these genes in males of *Cx. pipiens* and allow them to mate with female mosquitoes to observe post-copulatory physiological and behavioral effects such as blood feeding, fecundity and sperm survival in order to determine their functional roles. This research could enhance our understanding of how MAG genes influence female seasonal behavior changes and also potentially lead to novel mosquito control methods that rely on releasing sterilized or genetically modified male mosquitoes.

2. Methods

2.1 Mosquito Rearing

Cx. pipiens mosquitoes (Buckeye strain) were reared under the same dietary conditions from hatching. Mosquitoes were reared at 18°C under either long-day conditions with 16h light and 8h dark to induce summer-like phenotypes or short-day conditions with 8h light and 16h dark to promote diapause initiation in females and potentially induce males to express genes associated with sperm survival and storage. Adults were given access to a constant source of reverse osmosis water as well as 10% sucrose solution.

2.2 Confirming Differential Gene Expression between Long day and Short day-reared males

To confirm differential gene expression within the MAGs of long day and short day-reared male mosquitoes, live males were dissected in saline solution, and their accessory glands were removed (n=30 MAGs/sample; 5 samples/rearing condition) on the day of adult emergence. RNA was isolated from the tissue samples using TRIzol (Invitrogen) and complementary DNA (cDNA) was synthesized using the Maxima First Strand kit (Thermo Scientific) according to the manufacturer's protocol.

The expression of our target genes (*CapB, Tryp1* and *GST*) was measured and compared to reference genes (*Rp49, RpL19 and 28S*) using quantitative real-time PCR according to previously published protocols (Meuti et al. 2015). Previously published primer sequences for *Rp49* (Gentile et. al 2006), *RpL19* (Zhang and Denlinger 2011), and 28S (Sim and Denlinger 2008) were used. Primers of *CapB* (CPIJ001240-RA) and *Tryp1* (CPIJ018529-RA) were designed using *Primer3* by inputting sequences for each gene. Standard curves were used to

evaluate all primers according to MIQE guidelines (Bustin et al. 2009; Table 1). qRT-PCR was performed in triplicate in a 96-well plate using reactions of 10 µL containing 5 µL iTaq Universal SYBR green PCR Master Mix, 400 nM of each primer and 1 µL cDNA.

The qRT-PCR data were analyzed by comparing relative fluorescence of our target genes and reference genes. The average cycle threshold (CT) across technical replicates of target genes was normalized to the average CT of the three reference genes within the same biological replicate by subtracting the reference gene CT from the target gene CT. Relative gene expression was then found by using the $2^{-\Delta CT}$ method and further normalized by dividing the relative expression by the average relative transcript levels in either short day-reared MAGs (*CapB* and *Tryp1*) or long day-reared MAGs (*GST*).

Primer Name	Primer Sequence	Target Gene	Melting Temp (°C)	R^2	Primer Efficiency	# Points on std curve
Cp_CapB_qFw1	TACTGGCTGTGTGCCAACTC	*CapB*	59.90	0.96	87.22%	6
Cp_CapB_qRev1	GTCCTCGACTCCCACATGAT		59.93			
Cp_GST_qFw1	GAGTACCACCATCCGCAGTT	*GST*	60.00	0.98	131.28%	6
Cp_GST_qRev1	GAGTTCAGCAGCTCCAATCC		59.96			
Cp_Tryp1_qFw1	GCAACGGGTAAGAAAGTCCA	*Tryp*	60.11	0.98	94.11%	4
Cp_Tryp1_qRev1	ATTCCAACGAATTCCACCAG		59.80			

Table 1: Sequences, melting temperatures, efficiencies and R^2 values of primers used to amplify *CapB, GST* and *Tryp1*

2.3 dsRNA Synthesis and Injection

Double stranded RNA (dsRNA) that was complementary in sequence to each of our target genes (*Tryp1, CapB, GST*) or *β-galactosidase* (*β-gal*; negative control) was synthesized using T7 RiboMax Express RNAi kit (Promega) according to the manufacturer's instructions.

Primers, without the T7 promoter are shown in Table 2, and while previously published primers were used to synthesize *β-gal* dsRNA (Meuti et al. 2015). Mosquitoes were reared once again under either long or short-day conditions at 18°C and were separated by sex upon pupation. On the day of adult emergence, long and short day-reared male mosquitoes were injected in the thorax and received ~3 ug of dsRNA (2 injections of 0.069 μL with a concentration of 21.74 μg/μL) specific to each gene (n = 170-195 males/dsRNA and rearing condition). Two days later, 20 males per treatment were euthanized to confirm whether the dsRNA treatment effectively reduced the expression of the gene of interest by comparing the relative expression of the target gene to its expression in *β-gal* controls using qRT-PCR. This time, total RNA was isolated from the bodies of 4 dsRNA injected males/biological replicate (5 replicates/dsRNA treatment and rearing condition) using TRIzol according to the manufacturer's instructions. cDNA synthesis and qRT-PCR reaction conditions were performed as above. However, here expression was only normalized to a single reference gene (*Rp49*) and gene expression was further normalized by dividing by the mean relative expression of each gene of interest in *β-gal* controls (Meuti et al. 2015; Chang and Meuti 2020).

Primer Name	Primer Sequence	Target Gene	Melting Temp (°C)
Cp_CapB_RNAi_Fw	CTGGTTGCTGAGGTGTACGA	*CapB*	59.9
Cp_CapB_RNAi_Rev	CTACAGCCAGGACCTCAAGC		60.01
Cp_Gst_RNAi_Fw	TGACAATTGCCGATTTGTGT	*GST*	59.97
Cp_Gst_RNAi_Rev	CTGGACGGTCAAACCTTTGT		60.01
Cp_Tryp1_RNAi_Fw	GATCGGCCTAAGGAAACCAT	*Tryp1*	60.29
Cp_Tryp1_RNAi_Rev	AAATCGTTGGAGTGGTGTCC		59.83

Table 2: Sequences and melting temperatures of primers, without the T7 promoter, used to synthesize dsRNA

2.4 Determining the role of Tryp1 and CapB on female fecundity

One week after adult emergence and injection of males, long-day reared virgin females of *Cx. pipiens* were placed in cages with long-day-reared males that had been injected with either *Tryp1, CapB* or *β-gal* in a 1:1 ratio (n = 41 to 53). One week later, female mosquitoes were provided access to chicken blood (Pel-Freeze, Biologicals) via an artificial blood feeder (Hemotek, UK) for 2 hours and again 2 days later. The number of females that consumed blood in each cage was recorded. One week later, oviposition water was added to the cages and the number of total egg rafts as well as the number of eggs in each raft were counted. The number of larvae that hatched were counted daily.

2.5 Data Analysis

All graphs and statistical analyses were done in Excel, R (1.4.1106) and/or Prism 9. Significant differences in the relative expression of genes of interest between long and short day-reared males, and between *β-gal* and dsRNA specific to the genes of interest were assessed using a Student's T-test. Significant differences in the number of females that consumed a blood meal between females that mated with control (*β-gal* dsRNA-injected) males and target (*Tryp1* or *CapB)* dsRNA-injected males were analyzed using a GLM, while significant differences in the number eggs that were laid, the number of larvae that were produced and hatch rate were measured with a one-way ANOVA.

Results

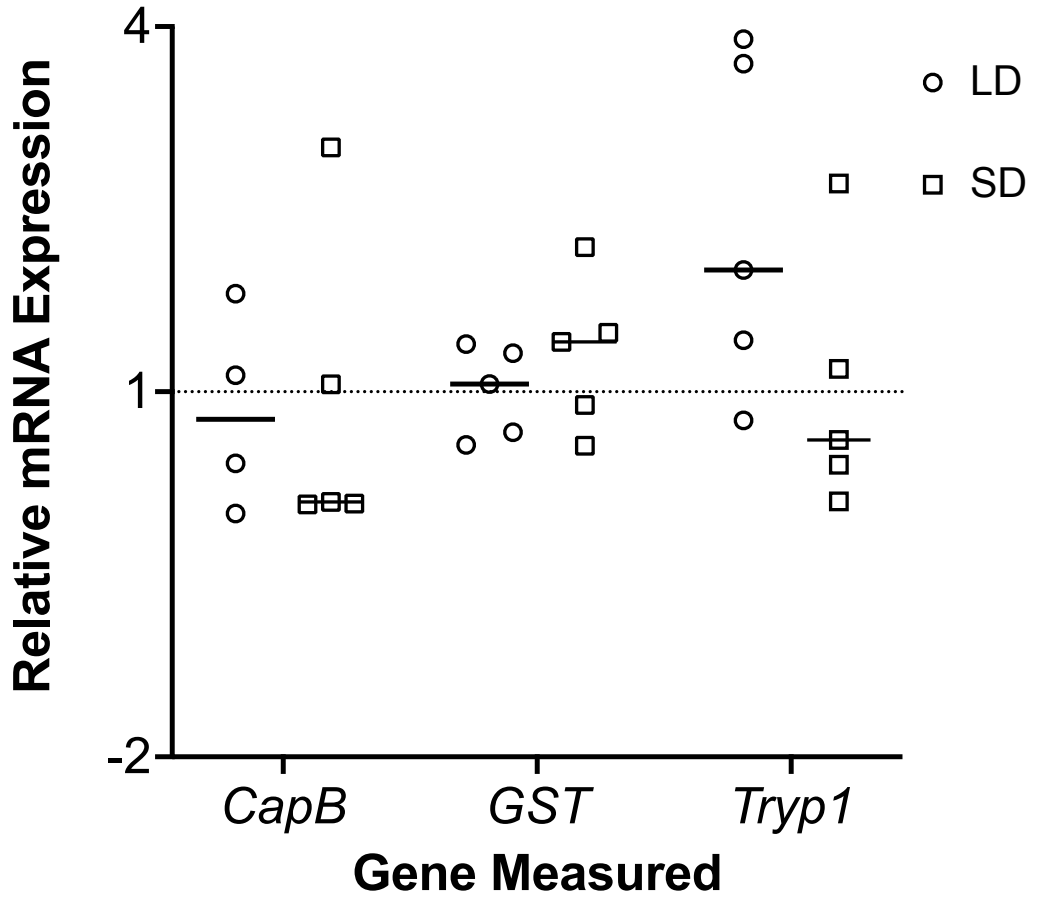

Figure 1: Relative expression of target genes (*CapB, GST, Tryp1*) in long day (LD) vs. short day (SD) reared males. Circles depict replicates from LD rearing conditions while squares depict replicates from SD rearing conditions. Horizontal bars represent median values. There were no significant differences in gene expression.

Comparison between long day-reared males and short day-reared males did not reveal any significant differences in *CapB, Tryp1,* or *GST* expression. The difference in mean relative expression of *CapB* between LD reared males and SD reared males ($8.46e^{-5}$) was not significant (Fig 1; p=0.36). Likewise, *Tryp1* did not show significant differences in expression between long day and short day-reared males (p=0.12). Increased *GST* expression in SD males compared to LD males (mean relative expression difference of 0.03) was not significant (p=0.37).

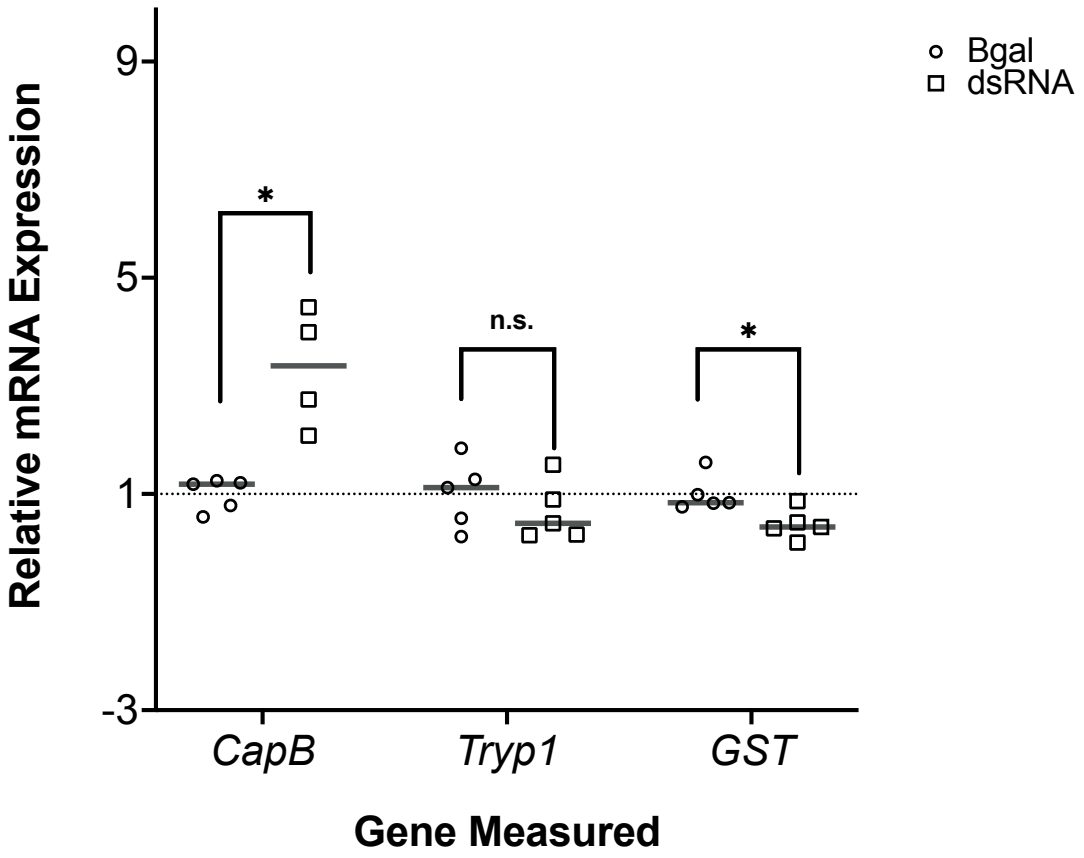

Figure 2: Relative expression of target genes (*CapB, Tryp1*and *GST*) in males that were injected with dsRNA normalized to the control group. Circles represent *β gal*-dsRNA injected control replicates, while squares represent replicates that were injected with dsRNA for the gene in that column. Horizontal bars represent median values and asterisks significant differences in gene expression.

RT-qPCR analysis of males that were killed post-injection revealed that dsRNA treatment affected the expression of some, but not all, of our target genes. Surprisingly, injecting males with *CapB* dsRNA resulted in a 4.6-fold increase in relative expression when compared to *β-gal* injected males, and that difference was significant (Fig 2; p=0.03). *GST* expression was significantly reduced by 40% in *GST* dsRNA-treated males (p=0.02). Injecting males with *Tryp1* dsRNA did not significantly change the level of *Tryp1* mRNA in comparison to *β-gal* dsRNA-treated males (p=0.42).

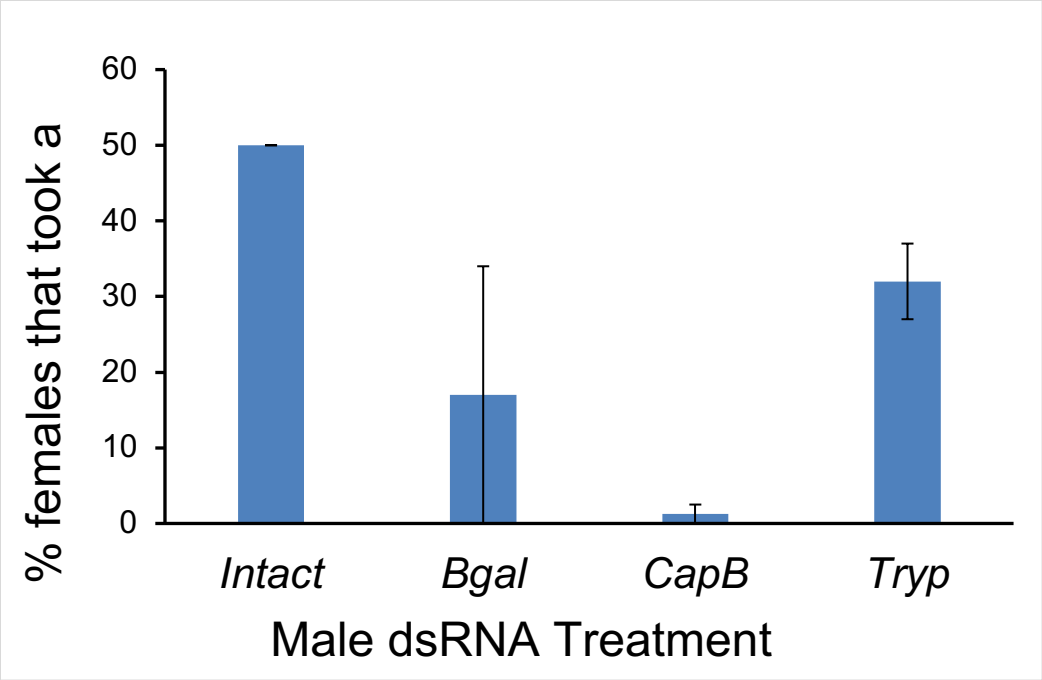

Figure 3: Effects of male-derived seminal fluid proteins (*CapB* and *Tryp1*) on female blood feeding relative to intact and *β-gal* dsRNA-injected controls. Blue bars represent the percent of females (n= 32 to 53) that took a blood meal after mating. Error bars represent standard error between 2 trials.

Females across all dsRNA treatment groups were less likely to take a blood meal than females mated with intact, un-injected males. Overall, 50.0% of females that mated with intact males took a blood meal while 17.0% of females that mated with *β-gal* dsRNA-injected (p= 0.12) and 32.0% of females that mated with *Trpy1* dsRNA-injected males took a blood meal (Fig. 3; p= 0.30). Females that mated with *CapB* dsRNA-injected males were the least likely to take a blood meal (1.3%) which was significantly lower than the intact group ($p<0.001$) and females that mated with *β-gal* dsRNA-injected males (p=0.04).

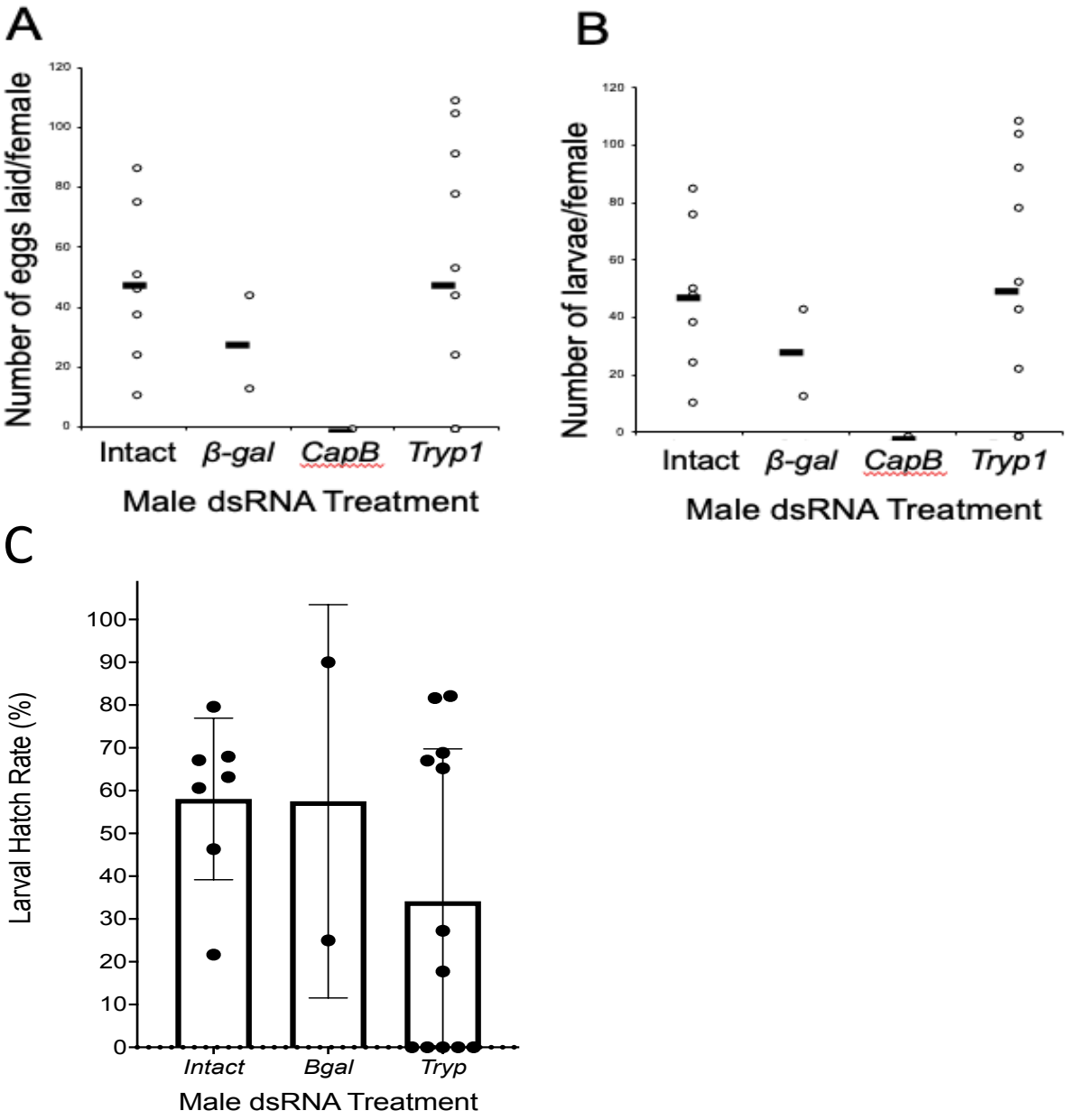

Figure 4: Effects of male-derived seminal fluid proteins (*CapB* and *Tryp1*) on female fecundity relative to intact and *β-gal* dsRNA-injected controls. Individual points represent number of eggs/raft laid (a) and number of larvae/egg raft (b) while horizontal bars (a and b) represent median values. Individual points represent hatch rate of each egg raft laid by a given treatment group, while rectangles represent mean hatch rate and error bars represent standard error (c).

Injecting males with *CapB* dsRNA had significant effects on female egg laying and larval hatching, while injecting males with *Tryp1* dsRNA did not. Females that mated with intact males produced an average of 92 ± 20 eggs/raft (Fig 4a), 49 ± 10 larvae/egg raft (Fig 4b), and the average hatch rate for intact-mated females was 58.0% (Fig 4c). Females mated with *β-gal* dsRNA-injected males produced an average of 55 ± 5 eggs/egg raft, 30 ± 15 larvae/egg raft and average hatch rate was 57.5%. Females that mated with *Tryp1* dsRNA-injected males laid 102 ± 15 eggs/egg raft, which was not significantly different from the number of eggs laid by females who mated with intact males (p=0.99) and *β-gal* dsRNA-injected males (p=0.82). However, females that mated with *Tryp1* dsRNA-injected males did lay egg rafts that broke apart more easily than other treatment groups (Fig 5). Females that mated with *Tryp1* dsRNA-injected males produced an average of 43 ± 14 larvae/egg raft which was not significantly different from females that mated with intact (p=0.99) or *β-gal* dsRNA-injected males (p=0.88). The *Tryp1* treatment group appeared to have a lower hatch rate than the control group (34 ± 35 larvae/egg raft). However, these results were not significant (p=0.61). Females from the *CapB* treatment group did not lay any eggs or hatch any larvae.

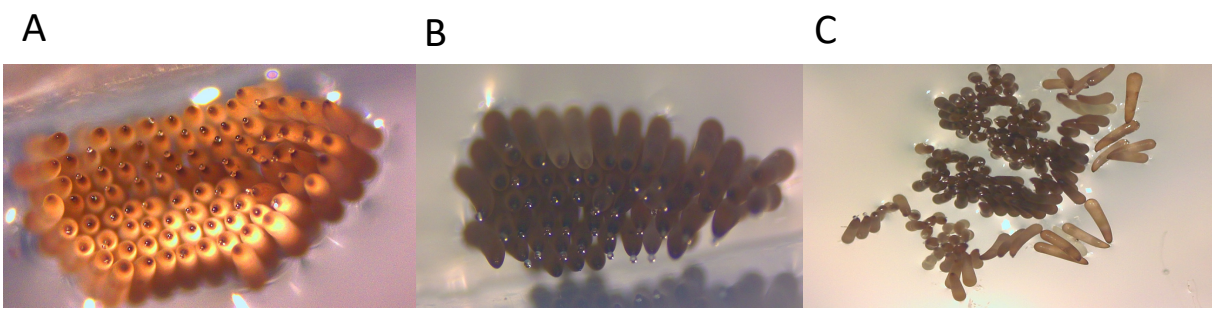

Figure 5: Egg rafts laid by females mated with intact males (a), control group males injected with *β-gal* dsRNA (b) and males injected with *Tryp1* dsRNA (c). Apparent color differences are due to lighting differences at the time of photo capture. Egg rafts from the *Tryp1* treatment group broke apart more easily than intact and control groups.

Discussion

Our experiments suggest that MAG proteins affect female behavior and physiology. No significant differences were found in the expression of *CapB, Tryp1* or *GST* between long day and short day-reared males. Females that mated with *CapB* dsRNA-treated males were less likely to take a blood meal and failed to lay any eggs or produce larvae, whereas females mated with *Tryp1* dsRNA-treated males laid egg rafts that broke apart more easily than eggs laid by females that had mated with intact and *β-gal* dsRNA-injected males, but *Tryp1* dsRNA-treated males did not significantly affect female blood feeding or egg laying and hatching.

Surprisingly, we found no significant differences in relative gene expression for our target genes between long day and short day-reared males. The average relative expression for both *CapB* and *Tryp1* were low for both sets of rearing conditions, which may indicate that these genes were not abundantly expressed across the tested generation of males. Additionally, a subsequent re-analysis of the RNAseq dataset that initially showed these genes were differentially expressed in the MAGs of long and short-day reared males failed to show differential expression of these genes. Further research should be directed toward understanding seasonal changes in MAG gene expression.

Injecting males with dsRNA had variable effects. Interestingly, we found that *CapB* caused a significant increase in the expression of the gene. Generally, dsRNA is used to silence genes, but Kulkarni et. al (2006) explains that dsRNA can have unintended effects and lead to false positive or false negative effects on genes in *D. melanogaster*. An analysis by Munkácsy et al. (2016) also revealed that injecting dsRNA can cause unintended gene up-regulation in a small percentage of cases. We saw no significant change in the level of *Tryp1* transcripts in dsRNA-treated males. Future work should be done to synthesize *Tryp1* dsRNA that effectively knocks

down mRNA expression, as well as to test the effects of suppressed MAG-derived *Tryp1* on female biting behavior, egg laying, and larval hatching in *Cx. pipiens.*

We found that females that mated with *CapB* dsRNA-injected males were less likely to take a blood meal. This result is inconsistent with previous findings that these proteins are expressed in *Ae. aegypti* and *Ae. albopictus* females after blood feeding and affect blood meal digestion (Sirot et al. 2011; Boes et al. 2014). Injecting *Tryp1* dsRNA in males did not negatively affect blood feeding behavior and had an insignificant effect on the number of eggs laid and larvae hatched. According to Müller (1995), and Dana et al. (2005), some Trypsin proteins are found to be readily expressed in females before blood feeding, while some are only expressed after taking a blood meal. Their studies suggest that females transcribe *trypsin* mRNAs on their own and would still consume and digest blood meals without receiving these proteins from males during copulation.

Surprisingly, we discovered that females who mated with *Tryp1* dsRNA-treated males produced similar amounts of eggs and larvae when compared to other treatments. However, females that mated with the *Tryp1* dsRNA-injected males did lay egg rafts that were less stable and more likely to fall apart when compared to other treatment groups. Previous experiments in *Ae. aegypti* have shown that injecting Trypsin inhibitors into female mosquitoes leads to impaired egg development (Borovsky 1988, Borovsky and Mahmood 1995). These results are not consistent with our data, as slightly more eggs were lain by females who mated with *Tryp1* dsRNA-treated males, indicating that egg development was not affected. However, since females that mated with *Tryp1* dsRNA-treated males laid egg rafts that more easily fell apart, *Tryp1* may affect adherence of eggs to one another in a raft. Further experiments would need to be conducted in order to better understand the role of *Tryp1* on egg adhesion in *Cx. pipiens.*

Our results indicate that differences in MAG gene expression potentially facilitate seasonal differences in female physiology and behavior after mating. Female *Cx. pipiens* were less likely to take a blood meal if they mated with *CapB* dsRNA-injected males. Although injecting males with *Tryp1* dsRNA did not have effects on blood feeding and total eggs laid or larvae hatched, that treatment group did have a lower hatch rate when compared to other treatment groups. Future work should be directed toward identifying functional effects of other MAG-derived seminal proteins on female reproductive physiology and behavior. The results of this study strengthen the argument for the use of genetically modified male mosquitoes to serve as targets for mosquito population and disease control (reviewed by Flores and O'Neill 2018). Modifying male mosquitos in the laboratory to knock down *Tryp1* and releasing them could lead to wild female mosquitoes laying egg rafts that frequently fall apart which could potentially reduce larval hatch rate, thereby reducing wild mosquito populations. Similarly, releasing males engineered to have altered *CapB* expression could lead to a decrease in blood feeding behavior in wild female populations, also driving down population numbers and decreasing disease transmission.

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